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Pharmacological Profile of SB 203580, a Selective Inhibitor of Cytokine Suppressive Binding Protein/p38 Kinase, in Animal Models of Arthritis, Bone Resorption, Endotoxin Shock and Immune Function

ALISON M. BADGER, JEREMY N. BRADBEER, BART VOTTA, JOHN C. LEE, JERRY L. ADAMS and DON E. GRISWOLD

Departments of Cellular Biochemistry (A.M.B., J.N.B., B.V., J.C.L.), Immunopharmacology (D.E.G.) and Medicinal Chemistry (J.L.A.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania

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ABSTRACT

SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole], a selective cytokine suppressive binding protein/p38 kinase inhibitor, was evaluated in several models of cytokine inhibition and inflammatory disease. It was demonstrated clearly to be a potent inhibitor of inflammatory cytokine production *in vivo* in both mice and rats with IC_{50} values of 15 to 25 mg/kg. SB 203580 possessed therapeutic activity in collagen-induced arthritis in DBA/LACJ mice with a dose of 50 mg/kg resulting in significant inhibition of paw inflammation and serum amyloid protein levels. Antiarthritic activity was also observed in adjuvant-induced arthritis in the Lewis rat when SB 203580 was administered p.o. at 30 and 60 mg/kg. Evidence for disease-modifying activity in this model was indicated by an improvement in bone mineral density and by histological evaluation. Additional evidence for beneficial effects on bone resorption was provided in the fetal rat long bone assay in which SB 203580 inhibited ^{45}Ca release with an IC_{50} of 0.6 μM. In keeping with the inhibitory effects on lipopolysaccharide-induced tumor necrosis factor-α in mice, SB 203580 was found to reduce mortality in a murine model of endotoxin-induced shock. In immune function studies in mice treated with SB 203580 (60 mg/kg/day for 2 weeks), there was some suppression of an antibody response to ovalbumin, whereas cellular immune functions measured *ex vivo* were unaffected. This novel profile of activity strongly suggests that cytokine inhibitors could provide significant benefit in the therapy of chronic inflammatory disease.

Cytokines such as IL-1 and TNF-α play a predominant role during inflammatory responses and autoimmune disease (Dinarello, 1991). Evidence for their key participation in acute and chronic inflammation has been provided by the demonstration that protein antagonists such as IL-1ra and monoclonal antibodies to TNF-α, and its soluble receptor, can interfere with various acute and chronic inflammatory responses. Another approach to the control of proinflammatory cytokines is to inhibit their production, ideally through the use of p.o. active low molecular weight compounds. One class of compounds that is effective in this respect is the pyridinyl imidazoles which have been shown to inhibit cytokine production *in vitro*, and *in vivo* they can attenuate the inflammatory components of disease in the absence of generalized immunosuppression (Griswold *et al.*, 1988; Lee *et al.*, 1993; Reddy *et al.*, 1994).

SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] (fig. 1) is a member of a new series of pyridinyl imidazole compounds which inhibit IL-1 and TNF-α production from LPS-stimulated human monocytes and the human monocyte cell line THP-1 with IC_{50} values of 50 to 100 nM (Lee *et al.*, 1994a,b; Gallagher *et al.*, 1995). The term CSAID™ has been coined for these compounds and they have shown activity in a number of animal models of acute and chronic inflammation (Lee *et al.*, 1993). The molecular target of SB 203580 and related compounds has been identified as a pair of closely related mitogen-activated protein kinase homologs, alternatively termed CSBP (Lee *et al.*, 1994b), p38 (Han *et al.*, 1994) or RK (Rouse *et al.*, 1994). The binding of the CSAID™ compounds to the target CSBP in

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ABBREVIATIONS: IL, interleukin; TNF, tumor necrosis factor; LPS lipopolysaccharide; CSBP, cytokine suppressive binding protein; CO, cyclooxygenase; RAP, rapamycin; RPMI, Roswell Park Memorial Institute; Con A, concanavalin A; OVA, ovalbumin; CFA, complete Freund's adjuvant; SAP, serum amyloid protein; AA, adjuvant arthritis; BMD, bone mineral density; BMC, bone mineral content; DXA, dual X-ray absorptiometry; PTH, parathyroid hormone; gal, galactosamine; LO, 5-lipoxygenase.

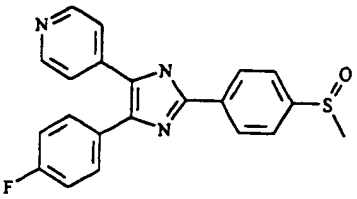


Fig. 1. Structure of SB 203580.

THP.1 cytosol correlates with their cytokine biosynthesis inhibition (Lee *et al.*, 1994b), indicating a role for CSBP in the production of cytokines in response to various stimuli (Lee and Young, 1996).

Compounds structurally related to SB 203580 have been tested previously in a number of animal models for their anti-inflammatory activity, including collagen-induced arthritis (Griswold *et al.*, 1988) and endotoxin shock (Badger *et al.*, 1989; Olivera *et al.*, 1992). These models are relatively insensitive to CO inhibitors, which adds credence to the cytokine suppressive nature of the CSAID™ molecules. In this manuscript, we show that cytokine inhibition with SB 203580 has beneficial effects in animal models of disease with only minor effects on immune function.

Materials and Methods

Animals. DBA/1 LACJ, BALB/c and C57BL/6 male mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male Lewis rats were obtained from Charles River Laboratories (Raleigh, NC.) Within any given experiment, only animals of the same age were used. All experimental procedures were in accordance with National Institutes of Health guidelines and were reviewed by the SmithKline Beecham Animal Care and Use Committee (King of Prussia, PA).

Materials. SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] was synthesized at SmithKline Beecham Pharmaceuticals (fig. 1). For *in vivo* assays, SB 203580 was administered p.o. in 0.03 N HCl-0.5% tragacanth (Sigma Chemical Co., St. Louis, MO) at the doses indicated. RAP was prepared by fermentation at SmithKline Beecham Pharmaceuticals (Brockham Park, UK). RPMI 1640 was obtained from Flow Laboratories (Rockville, MD and contained 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM L-glutamine (GIBCO, Grand Island, NY). This medium will be known as RPMI-10. Con A was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Endotoxin (LPS) was either *Escherichia coli*, type W or *Salmonella typhosa* (Difco Laboratories, Detroit MI) and OVA was from Sigma.

LPS-induced TNF production in mice and rats. BALB/c male mice in groups of three to five were treated with vehicle or compound by p.o. gavage and 30 min later the animals were injected i.p. with 25 μg/mouse of endotoxin (*E. coli*, type W, Difco). Two hours later, the animals were euthanized by carbon dioxide asphyxiation and plasma was obtained from individual animals by collecting blood into heparinized tubes. The samples were clarified by centrifugation at 12,500 × *g* for 5 min at 4°C. The supernatants were decanted to new tubes (may be stored at −20°C) and were assayed for mouse TNF-α by ELISA (Olivera *et al.*, 1992). The range of sensitivity of the ELISA is 25 to 800 pg/ml of mouse TNF-α. For the induction of TNF-α in Lewis rats, the animals were treated with SB 203580 30 min before the injection of LPS (30 μg/kg i.p.). TNF-α levels were measured 90 min later by ELISA.

Collagen-induced arthritis. Type II collagen arthritis was induced in male DBA/1 LACJ mice (30–35 g, Jackson Laboratories) by the method of Wooley (1988). The mice were primed with an emulsion consisting of CFA (Difco Laboratories) combined with an equal volume of a freshly prepared solution of 2.0 mg of collagen type II (bovine nasal septum, Elastin Products Co., Inc., Owensville, MO) per ml of 0.01 N acetic acid. Extra *Mycobacterium butyricum* (Difco) was added to the CFA to make the concentration twice that present in the commercial preparation. The CFA/collagen emulsion was prepared by mixing through two connected 20-ml syringes. An intradermal injection of 0.1 ml of emulsion per mouse was administered at the base of the tail. Twenty-one days later, the mice were boosted by an i.p. injection of 0.1 ml of freshly prepared 1.0 mg of bovine collagen II/ml of 0.01 N acetic acid per mouse. Joint swelling presented within a few days and the mice were evaluated for incidence and severity of inflammation, assigned randomly to study groups, ear tagged and the individual mouse's dosing regimen was begun. Severity of joint swelling was determined subjectively for each limb by using a scale of 1 (one or more phlanges per limb) to 4 (maximum swelling per limb). A severity score of at least 2 on one limb (excluding phlanges) was required for an animal to be assigned to a study group. Before dosing, each mouse was bled by the tail vein for a serum sample (100–150 μl of blood). Disease severity was assessed on days 7 and 10 after which blood was collected (tail vein on day 7 and by exsanguination on day 10) for serum. The serum samples were assayed for mouse SAP by using a radio immunologically quantitated Western blot method (Griswold *et al.*, 1988).

Statistical analysis. Clinical severity and levels of SAP and TNF-α were analyzed by using the Student's *t* test, with P values less than .05 considered significant.

AA. AA was induced by a single injection of 0.75 mg of *M. butyricum* (Difco) suspended in paraffin oil into the base of the tail of male Lewis rats, 6 to 8 weeks old (160–180 g). Hindpaw volumes were measured by a water displacement method on day 16 and/or day 22. Test compounds were homogenized in acidified 0.5% tragacanth (Sigma) and were administered p.o. in a volume of 10 ml/kg. Control animals were administered vehicle (tragacanth) alone.

Percentage of inhibition of hindpaw lesions was calculated as follows:

$$\% \text{ Inhibition} = 1 - \frac{\text{AA (Treated)}}{\text{AA (Normal)}} \times 100$$

For statistical analysis, paw volumes of rats treated with SB 203580 were compared to the untreated controls by Student's *t* test.

BMD, as well as BMC and bone area were determined for the distal tibia by DXA by using the Hologic QDR-1000 equipped with high resolution scanning software as we have described previously (Bradbeer *et al.*, 1996).

Tibio-tarsal joints from representative animals from the following three groups of rats were examined histologically; normal rats, AA control rats and AA rats treated with SB 203580 at 60 mg/kg/day. Rats were sacrificed by CO_2 administration and the rear legs were fixed in formalin, decalcified in formic acid and the feet removed from the legs at the distal tibial diaphysis. After routine processing, the feet were embedded and coronal sections were cut in the plane midway through the tibiotarsal and tarsotarsal joints. Sections were stained with Safranin O and counterstained with fast green.

Bioassay for IL-6. Serum samples were obtained when the rats were euthanized. IL-6 levels were determined by using the previously described B9 bioassay (Aarden *et al.*, 1985). Briefly, B9 cells (5×10^3 cells/well in 96-well flat-bottomed plates) were cultured at 37°C with serial dilutions of rat serum in a final volume of 100 μl of RPMI-10. After 68 hr, 0.5 μCi of [^{3}H]thymidine was added and was incubated for 6 hr at 37°C. Cells were harvested and radioactivity incorporation was determined. IL-6 was quantified from a standard curve including known amounts of rat IL-6 (0.1–100 pg/ml). B9 proliferation was unaffected by any agents used in this study.

Fetal rat long bone resorption assay. This assay was performed essentially as described previously (Raisz, 1965; Stern and Raisz, 1979). Timed-pregnant Sprague Dawley rats (Taconic Farms, Germantown, NY) were injected s.c. with 200 μCi of $^{45}CaCl_2$ on day 18 of gestation, housed overnight, then anesthetized with Inovar-Vet (Pittman-Moore, Mundelein, IL) and sacrificed by cervical dislo-

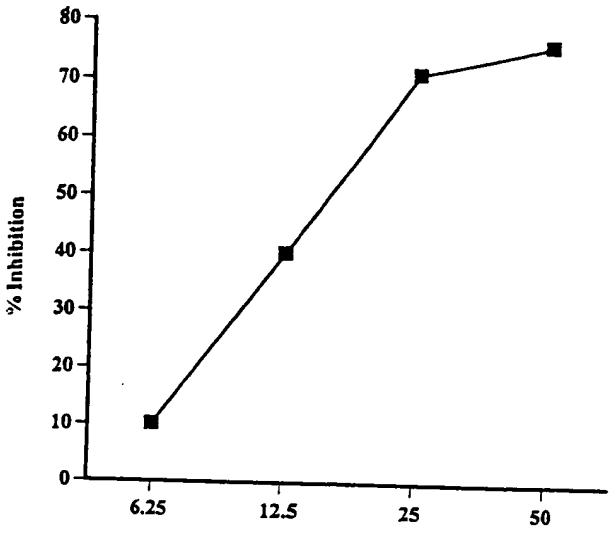


Fig. 2. Inhibition of plasma TNF levels in BALB/c mice. SB 203580 was administered 30 min before LPS challenge and TNF was measured by ELISA 2 hr later. Data are presented as percentage of inhibition by using three to five animals per group. Significant inhibition was observed at 50, 25 and 12.5 mg/kg ($P < .001$) with an IC_{50} of 15 mg/kg.

cation. Fetuses were removed aseptically and radii and ulnae were dissected free of surrounding soft tissue and cartilaginous ends. The bones were cultured 18 to 24 hr in BGJ_b medium (Sigma) containing 1 mg/ml of bovine serum albumin, then were transferred to fresh medium and cultured for an additional 48 hr in the absence or presence of 50 ng/ml of PTH (human, 1–34) and test compound. Calcium released into the medium and total residual calcium in the bones were measured by liquid scintillation spectrometry. Data are expressed as the percentage of calcium released from treated bones as compared to corresponding control bones. Statistical differences were assessed by using a one-way analysis of variance for nonpaired samples. Data are presented as mean ± S.E.

Endotoxin shock. Pathogen-free male C57BL/6 mice were obtained from Jackson Laboratories. Age-matched mice, 6 to 12 weeks old, were used. This model of shock was performed as described previously (Badger *et al.*, 1989; Olivera *et al.*, 1992). Briefly, 0.1 μg of LPS from *Salmonella typhosa* (Difco) mixed with D-(+)-gal (Sigma: 500 mg/kg) was injected i.v. in 0.25 ml of pyrogen-free saline (this mixture is referred to as LPS/D-gal). Compounds to be tested were administered p.o. 30 min before the i.v. injection of LPS/D-gal. Blood was collected *via* cardiac puncture 1 hr after LPS/D-gal and serum samples were stored at −20°C until evaluation for TNF-α by ELISA. Survival was monitored, in separate groups of animals, for 48 hr after LPS challenge, at which time no further deaths occurred in either treated or untreated control mice.

Immune function assays. Female BALB/c mice were immunized with 100 μg of OVA in 50 μl of CFA in both hind footpads (OVA was prepared at 4 mg/ml and diluted 1:1 in CFA). Mice were then treated for 5 days a week for 2 weeks with 60 mg/kg of SB 203580 or 50 mg/kg of RAP administered i.p. in a vehicle composed of 10% ethanol, 10% cremophor and 80% saline. At the termination of the experiment (day 12), spleen and lymph nodes were harvested and cell suspensions were prepared by standard procedures. For the response to OVA and Con A, lymph node cells (5×10^5) were established in 96-well round bottomed plates in the presence or absence of serially diluted Con A or OVA for 72 hr. For the mixed lymphocyte reaction, cells from treated BALB/c mice (1×10^6) were established in 96-well flat bottomed plates along with C57BL/6-irradiated (3000 R) stimulator cells (1×10^6). Cell cultures were incubated at 37°C/5% CO_2, with 0.5 μCi of [^{3}H]thymidine added for the last 18 hr of culture. Cell-associated radioactivity was measured after collection onto glass-fiber filters by scintillation counting. For OVA-specific antibody response, sera from immunized mice were tested for activity by ELISA which has been described in detail previously (Reddy *et al.*, 1994).

Results

Inhibition of TNF-α and collagen-induced arthritis in mice. Demonstration of the ability of SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] to inhibit inflammatory cytokine production *in vivo* was accomplished by using BALB/c mice challenged with LPS (25 μg i.p.). As seen in figure 2, SB 203580 given p.o. 30 min before LPS challenge inhibited the production of TNF-α (ED_{50}, 15 mg/kg p.o.).

Given the potent ability of SB 203580 to inhibit TNF-α production *in vivo*, it was of interest to evaluate the effect of the compound on a chronic inflammatory model. Collagen-induced arthritis was induced in DBA/1 LACJ mice by injection of bovine Type II collagen in CFA at the base of the tail, followed 21 days later by a booster injection of collagen solubilized in acetic acid (i.p.). Animals with significant disease were treated with SB 203580 (50 mg/kg p.o., b.i.d.). At the end of 7 days, the disease severity was judged on a scale of 0 to 4$^+$ and blood was obtained for analysis of serum amyloid P component. As seen in table 1, in two separate studies, SB 203580 significantly reduced disease severity (72%, $P < .01$ and 45%, $P < .05$, respectively) as well as acute phase reactant (SAP) levels (42%, $P < .05$ and 52%, $P < .001$, respectively).

Inhibition of TNF-α and AA in rats. TNF-α was also inhibited in SB 203580-treated Lewis rats. This was shown by treating normal rats with SB 203580 p.o. 30 min before a challenge with 30 mg/kg of LPS i.p. Plasma TNF-α levels measured 90 min later were inhibited by 53% at 25 mg/kg ($P < .01$) and by 38% at 12.5 mg/kg ($P < .01$) with no inhibition observed at 6.2 mg/kg (table 2).

In the rat model of AA, p.o. administration of SB 203580

TABLE 1

Effect of SB 203580 on type II collagen-induced Arthritis in DBA/1 mice

This table summarizes two studies in which the mice were dosed for 7 days at 50 mg/kg (p.o., b.i.d.), after the animals had presented with paw or joint edema/swelling. The data are the mean ± S.E. from a group of vehicle (0.03 N HCl/0.5% Tragacanth) and SB 203580-treated controls. In Experiment II, the mice were entered into the study as fully complemented groups. Data are significantly different from the control: * $P < .05$; ** $P < .01$; *** $P < .001$.

Treatment	*n*	Severity Index	%	*n*	SAP	%
Experiment I						
Control	9	5.86 ± 1.17		9	189.89 ± 28.29	
SB 203580	8	1.63 ± 0.79	72**	8	110.66 ± 17.75	42*
Experiment II						
Control	10	5.75 ± 0.83		8	181.30 ± 10.36	
SB 203580	10	3.15 ± 0.51	45*	8	86.61 ± 6.23	52***

TABLE 2

Inhibition of LPS-stimulated TNF-α levels in SB 203580 Lewis rats

Rats were dosed (p.o.) 30 min before treatment with LPS (30 μg/kg/i.p.). Plasma TNF was measured 90 min after LPS administration. Data are mean ± S.E. for six animals per group. * P < .05; ** P < .01.

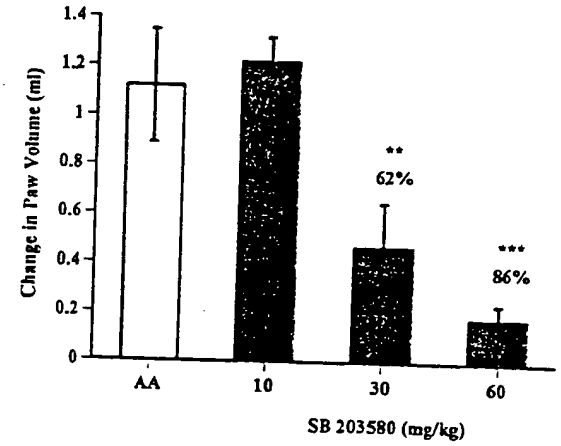
Treatment	TNF-α	% Inhibition
	ng/ml	
Control (untreated)	42.15 ± 5.05	
SB 203580		
25 mg/kg	19.91 ± 2.77	53**
12.5 mg/kg	26.20 ± 4.56	38*
6.25 mg/kg	36.76 ± 3.56	13 N.S.

(10, 30 and 60 mg/kg p.o.) from day 0 to day 22 inhibited the development of immune-mediated hindpaw inflammation. On day 16, there was 86% inhibition at 60 mg/kg (P < .001) and 62% inhibition at 30 mg/kg (P < .01), with no effect observed at 10 mg/kg (fig. 3A). By day 22, the anti-inflammatory effect had lessened somewhat with 60% (P < .001) and 45% (P < .01) inhibition at 60 and 30 mg/kg, respectively (fig. 3B).

The anti-inflammatory and antiarthritic activities of SB 203580 were evaluated further by examining the BMC and BMD of the distal tibia region in treated AA rats. On day 22 when the rats were euthanized, hindlimbs were examined by DXA. When compared with the AA controls, there was a significant normalization of BMD (31%, P < .01) and BMC (26%, P < .01) in the rats treated with 60 mg/kg/day of the compound, indicating a protective effect on inflammation-mediated bone destruction and/or a direct effect on bone resorption proximal to the inflamed joint (fig. 4, A and B).

Histology of the tibio-tarsal joint from a normal rat and from rats challenged with adjuvant and then treated with vehicle (AA control) or SB 203580 is shown in figure 5. In the AA control joint, all of the original bone and marrow has been replaced by granulation tissue and newly formed woven bone. Remnants of articular cartilage are evident and the

A.



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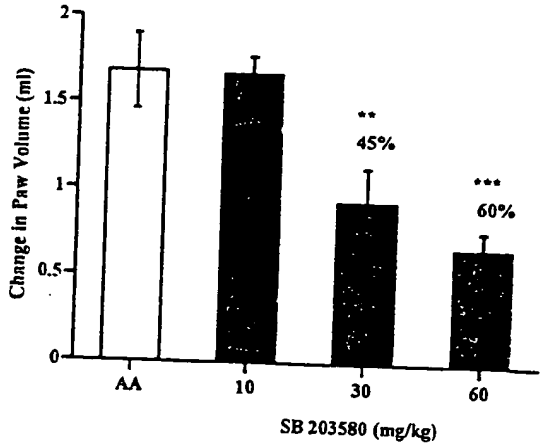
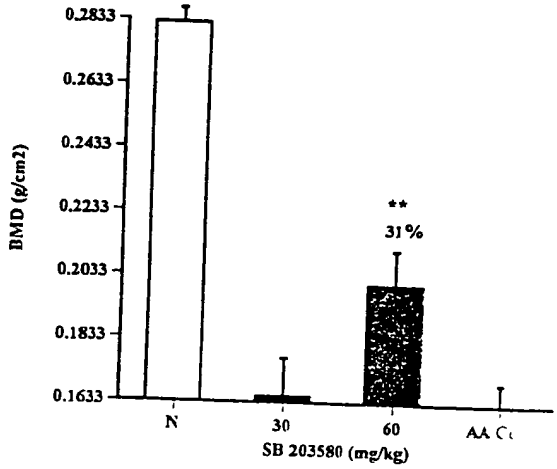


Fig. 3. Dose-dependent suppression of hindpaw inflammation in rats with AA by prophylactic administration of SB 203580 from days 0 to 22 (5 days a week). Paw inflammation was measured on day 16 (A) and on day 22 (B). Data are the mean and S.E.M. of 10 animals per group. **P < .01; ***P < .001, compared to the untreated AA controls.

A.



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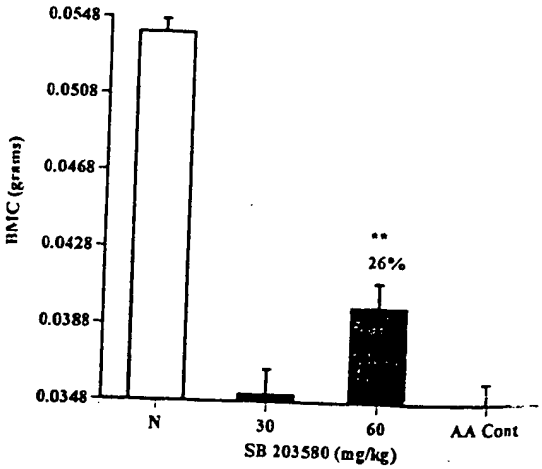

Fig. 4. Bone densitometry evaluation of the distal tibia in AA rats treated with SB 203580. Rats were treated with various doses 5 days a week from day 0 to day 22. Values are the percentage of normal (assigned a value of 100%), mean and S.E.M. of 10 animals per group. A, the BMD value was 0.2822 ± 0.0045 for normal rats and 0.01633 ± 0.0067 for AA rats, which is a 42% decrease in BMD in the diseased animals. B, the BMC value was 0.0540 ± 0.0007 for normal rats and 0.0348 ± 0.001 for AA rats which is a 36% decrease in BMC in the diseased animals. The effect of SB 203580 was statistically significant on both BMD and BMC. **P < .01.

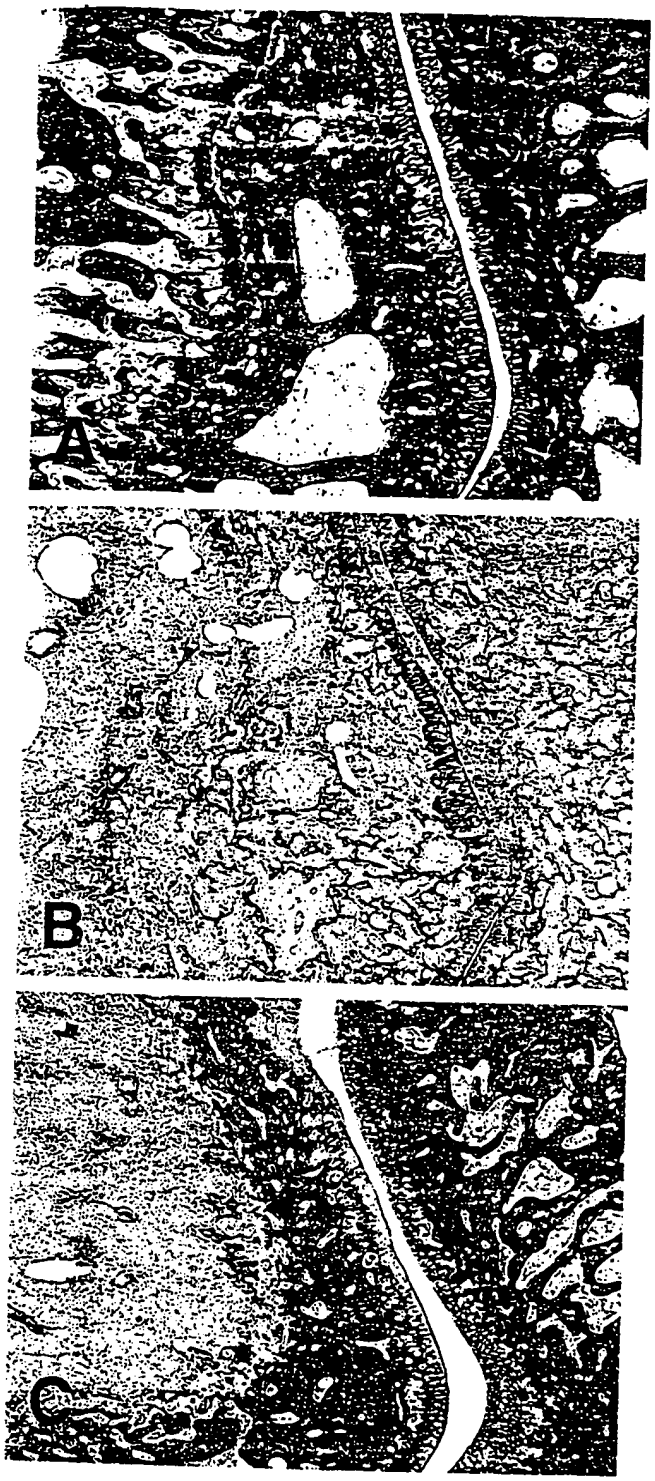

Fig. 5. A, photomicrograph of a normal tibio-tarsal joint. Cancellous bone of the metaphysis (left) is separated from the epiphysis (middle) by the growth plate (continuous pink line). The joint space between the tibia and tarsus (right) is clearly visible. B, photomicrograph of a tibio-tarsal joint typical of a rat suffering from AA. All of the original normal marrow and bone of the tibia and tarsus has been replaced by granulation tissue within which reactive woven bone has formed. The joint space (running vertically in approximately the same location as in the upper and lower panels) has been obliterated almost entirely by infiltrating granulation tissue and a large region of the articular cartilage on the tarsal side of the joint has been replaced by granulation tissue. C, photomicrograph of a tibio-tarsal joint from a rat which had been challenged with adjuvant and then treated with SB 203580 (60 mg/kg/day) for 22 days. Although a small amount of infiltration and cartilage erosion can be seen on the tibial side (top), the joint space and articular surface are otherwise normal. Whereas the cancellous bone of the tibial metaphysis has been lost and the marrow largely replaced by granulation tissue, woven bone has not yet formed. The bone and marrow of the tibial epiphysis and the tarsus are normal.

TABLE 3

Inhibition of serum IL-6 in AA rats treated with SB 203580

SB 203580 was administered orally 5× a week from day 0 to 22. Serum IL-6 was measured on day 23. Data are mean ± S.E. for 10 animals per group. *** $P < .001$.

Treatment	IL-6	% Inhibition
	ng/ml	
Control AA rats	1.85 ± 0.10	
SB 203580		
60 mg/kg	1.11 ± 0.13	40***
30 mg/kg	1.43 ± 0.10	23***
10 mg/kg	1.75 ± 0.07	5 N.S.

former joint space has been infiltrated with granulation tissue. The joint from the rat treated with SB 203580 shows protection of the joint space, articular surfaces and subchondral bone. Although the tibial metaphyseal cancellous bone and marrow have been replaced by granulation tissue, these components of the tarsus are normal. This histological appearance is consistent with SB 203580 having retarded the progression of the adjuvant-induced arthritic lesion.

Serum IL-6 levels in AA rats treated with SB 203580 on days 0 to 22 were measured in a B9 hybridoma proliferation assay. Normal rats had serum IL-6 levels of < 50 pg/ml, whereas levels in rats with untreated AA were elevated as high as 1.85 ng/ml. In rats treated with SB 203580, there was a 40% reduction in IL-6 at the 60 mg/kg dose ($P < .001$) and 23% inhibition at 30 mg/kg ($P < .001$) (table 3).

Fetal rat long bone assay. As studies in the AA rat showed clearly that treatment with SB 203580 had disease-modifying activity and protective effects on both bone and cartilage, we examined the effect of the compound in a fetal rat long bone resorption assay. In this assay, osteoclast-mediated bone resorption is monitored by measuring the release of ^{45}Ca into the culture medium from preradiolabeled fetal long bones. SB 203580 inhibited resorption in a concentration-dependent manner; 3 μM (85%, $P < .001$), 1 μM (80%, $P < .001$) and 0.3 μM (38%, $P < .05$). The IC_{50} was 0.6 μM (fig. 6).

Endotoxin shock. The effect of SB 203580 was evaluated in a mouse model of endotoxin shock. In this model, C57BL/6 mice are sensitized with D-(+)-gal, which makes them highly susceptible to the lethal effects of endotoxin (LPS). One hour before an i.v. injection of LPS/D-gal, control mice have serum levels of TNF-α up to 4 ng/ml. This is reduced in a dose-dependent manner by prophylactic administration of SB 203580 given 30 min before the injection of LPS/D-gal. Doses of 100, 50 and 25 mg/kg were active and inhibited TNF-α levels by 87% ($P < .001$), 62% ($P < .001$) and 42% ($P < .001$), respectively (table 4). In a separate group of mice that were monitored for survival, 84% of mice treated with the 100 mg/kg dose of SB 203580 survived compared to only 17% of control mice.

Immune function. In order to determine whether chronic administration of a CSAID™ molecule such as SB 203580 had detrimental (suppressive) effects on the immune system, BALB/c mice were immunized with OVA in CFA and then treated for 2 weeks (5 days a week) with 60 mg/kg i.p. of the

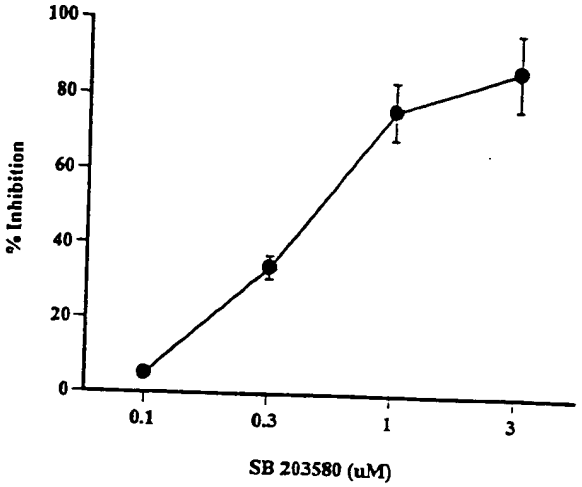

Fig. 6. SB 203580 inhibits PTH-stimulated fetal rat long bone resorption *in vitro* (IC_{50} = 0.6 μM). Fetal rat radii and ulnae (four bones per treatment per experiment) were cultured in the presence of 50 ng/ml of PTH and the indicated concentrations of SB 203580 for 48 hr as described under "Materials and Methods." Each data point represents mean and S.E.M. from three separate experiments. Bones cultured in the absence of PTH released approximately 14% of the incorporated ^{45}Ca. In the presence of PTH, control bones released approximately 45% of the incorporated ^{45}Ca.

compound or with RAP at 50 mg/kg. The serum antibody response of mice treated with RAP was suppressed totally by this treatment and there was a significant reduction in the anti-OVA serum antibody titer in mice treated with SB 203580 (fig. 7A). However, when lymph node cells from treated mice were examined for their response to the specific OVA antigen or to the mitogen Con A, no inhibition of proliferation was observed (fig. 7, B and C). Neither was there any inhibition of an allogeneic response in a mixed lymphocyte reaction between spleen cells from treated mice and C57BL/6 stimulator (3000 R) cells (fig. 7D). In all cases, the lymphocyte responses of RAP-treated mice were suppressed dramatically.

Discussion

The pyridinyl imidazoles are a novel class of compounds that have potent inhibitory effects on cytokine production both *in vitro* and *in vivo*, and also show anti-inflammatory activity in a variety of animal models (reviewed in Lee *et al.*, 1993). An early compound in this series, SK&F 86002, had cytokine suppressive activity (IC_{50}, 1 μM) (Lee *et al.*, 1993), but no significant antiproliferative activity (Reddy *et al.*, 1994). In addition to cytokine suppressive activity, SK&F 86002 and many structurally related analogs inhibited eicosanoid metabolism in LO and CO enzyme assays (Griswold *et al.*, 1987). In keeping with this profile of both cytokine and eicosanoid inhibition, SK&F 86002 and related compounds showed therapeutic activity in mouse collagen-induced arthritis (Griswold *et al.*, 1988) and carageenan-induced inflammation (Lee *et al.*, 1993), as well as analgesic activity in mouse abdominal constriction assays (Lee *et al.*, 1993). These activities, however, could not totally be attributed to LO/CO inhibition and the compounds clearly did not act as classical nonsteroidal anti-inflammatory drugs. Evidence for this was their activity in assays and models relatively insensitive to CO inhibition such as collagen-induced arthritis (Griswold *et al.*, 1988), the fetal rat long bone resorption assay (Votta and Bertolini, 1994) and mouse models of endotoxin shock (Badger *et al.*, 1989; Olivera *et al.*, 1992).

In studies designed to define the mechanism of cytokine suppression by the pyridinyl imidazoles, it was revealed that inhibition of TNF-α synthesis was primarily at the translational rather than the transcriptional level (Lee *et al.*, 1990; Young *et al.*, 1993), and that a block occurred before nascent peptide elongation (Young *et al.*, 1993; Prichett *et al.*, 1995; P. R. Young, unpublished data). Recent investigations using THP.1 cells, radiolabeled chemical probes for radioligand binding assays and photoaffinity labeling experiments have identified the molecular target of these compounds to be a pair of closely related mitogen-activated protein kinase homologs termed CSBPs (Lee *et al.*, 1994b). CSBP, alternatively termed p38 or RK, has subsequently been identified independently by several laboratories (Lee *et al.*, 1994b; Han *et al.*, 1994; Rouse *et al.*, 1994).

Inhibition of CSBP kinase activity by these compounds correlates with cytokine inhibition and THP.1 cytosol binding assays (Lee *et al.*, 1994b). SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole], a newer member of the pyridinyl imidazoles, is the best studied compound and has an IC_{50} of 0.22 μM as a CSBP inhibitor (Cuenda *et al.*, 1995; Gallagher *et al.*, 1995; T. F. Gallagher *et al.*, in press, 1996). The compound is highly specific for CSBP kinase with no inhibitory activity observed on a variety of other kinases (Cuenda *et al.*, 1995). A physiological substrate of CSBP is MAPKAP kinase-2, and SB 203580 inhibits the activation of this kinase and its subsequent phosphorylation of hsp 27 in stress-stimulated cells (Cuenda *et al.*, 1995). In *in vitro* monocyte cultures, SB 203580 inhibits IL-1 and TNF-α from LPS-stimulated human monocytes (IC_{50}, 50–100 nM) as well as the production of leukotriene B_4 from calcium ionophore (A23187)-stimulated human monocytes (IC_{50}, 1.5 μM) (M. D. Chabot-Fletcher, unpublished observations). In HL-60 cells, SB 203580 had little effect on the LO pathway,

TABLE 4

SB 203580 inhibits serum TNFα and improves survival in a murine model of endotoxin shock

Male C57BL/6 mice were treated p.o. with SB 203580, 30 min before LPS/D-gal given i.v. Serum TNF was measured 1 hr later. Data are mean and S.E.M. of three to five animals per group. Survival was monitored in a separate group of mice (six per group). * $P < .05$ by Fisher's exact test. ND = not done.

Treatment/Dose	TNF-α	% Inhibition	% Survival
	pg/ml		
Control	3789 ± 142		17
SB 203580			
100 mg/kg	490 ± 172	87***	84*
50 mg/kg	1444 ± 130	62***	20 N.S.
25 mg/kg	2248 ± 130	42***	ND
12.5 mg/kg	3514 ± 214	7 N.S.	ND

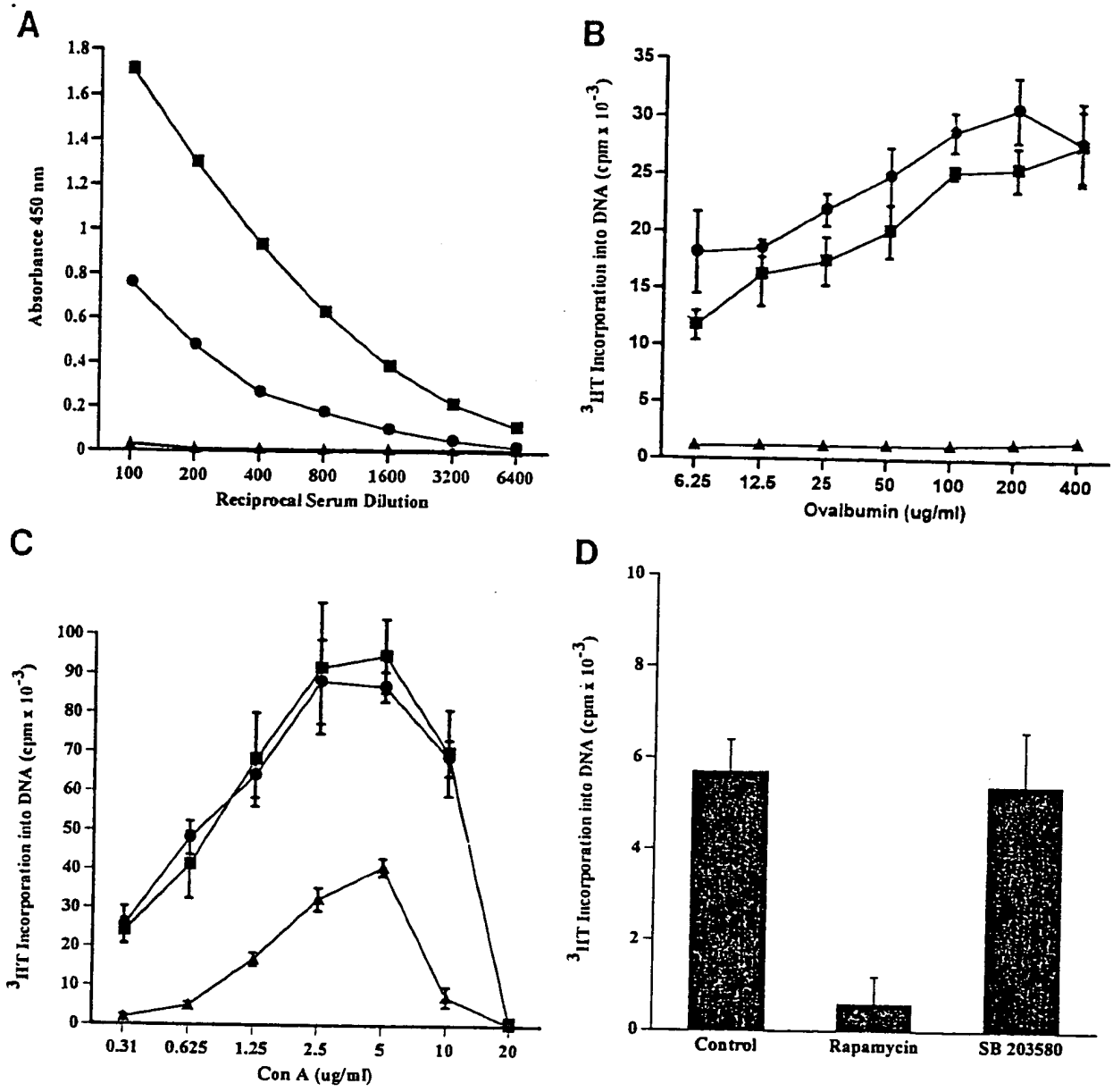

Fig. 7. Immune function in BALB/c mice treated with SB 203580 at 60 mg/kg i.p. for 2 weeks. Mice were immunized in the hind footpads with OVA in CFA as described under "Materials and Methods." A, serum antibody response to OVA measured in an ELISA assay. Data are from pooled serum samples. B, proliferative response of lymph node cells to varying doses of OVA. Data are mean and S.D. of six replicates. C, proliferative response of lymph node cells to varying doses of Con A. Data are mean and S.D. of six replicates. ■, control untreated; ●, SB 203580; ▲, RAP; and D, mixed lymphocyte reaction of treated BALB/c spleen cells against irradiated (3000 R) C57BL/6 stimulator cells. Data are mean and S.D. of six replicates.

but potently inhibited prostaglandin E_2 synthesis. SB 203580, however, had no direct inhibitory activity on pituitary growth hormone Synthase-1 and only modest inhibitory activity on LO (IC_{50}, 58 μM). The involvement of CSBP in the regulation of arachidonic acid availability, which is the rate limiting step for both LO and CO production, provides one mechanism which may explain these observations. In platelets, CSBP mediates the activation of cytosolic phospholipase A_2 by phosphorylation of cytosolic phospholipase A_2 in response to a thrombin agonist peptide (Kramer *et al.*, 1995). This activation was correlated with the subsequent release of arachidonic acid and formation of CO products. The observation that SB 203580 inhibits the synthesis of the inducible COX-2 enzyme provides an additional mechanism by which CSBP can regulate prostanoid synthesis (Lee *et al.*, 1994a).

In the studies reported in this paper, we have profiled SB 203580 in a number of pharmacological models both *in vitro* and *in vivo* and demonstrated its activity in a wide variety of TNF-α-mediated animal models. SB 203580 inhibited LPS-induced TNF-α *in vivo* in both mice and rats with IC_{50} values of 15 and 25 mg/kg, respectively. This inhibition of TNF-α was an indication that disease models such as mouse collagen-induced arthritis and rat adjuvant arthritis would be positively modulated by the compound. This was indeed the case and, in collagen-induced disease in DBA/1 LACJ mice, SB 203580 dosed for 7 days at 50 mg/kg p.o. (b.i.d.) reduced joint edema by 72 and 45% in two separate experiments. SAP, an acute inflammatory protein in mice, was also inhibited by 42 and 52%, respectively, in the two experiments. Evidence for the critical role of endogenous TNF-α in this disease model

has been provided by the observations that administration of anti-TNF-α antibodies can ameliorate the disease (Piguet *et al.*, 1992; Thorbecke *et al.*, 1992; Williams *et al.*, 1992) and that TNF-α transgenic mice spontaneously develop arthritis (Keffer *et al.*, 1991).

TNF-α clearly plays a proinflammatory role in another animal model of RA, the AA rat, in which elevated levels have been observed in the plasma and joints (DiMartino *et al.*, 1993; Smith-Oliver *et al.*, 1993). In this disease model, SB 203580 was very effective in reducing paw inflammation at doses of 30 and 60 mg/kg/day with optimum inhibition observed at 60 mg/kg/day (86% inhibition on day 16). Evidence for the protection of joint integrity at this dose was provided by the observation that there was a normalization of BMD (31%) and BMC (26%) as measured by DXA. This was also reflected in the histological evaluation of the affected joints, in which a clear beneficial effect was observed on both bone and cartilage. In keeping with the compound's disease-modifying activity, our studies also demonstrated that serum levels of IL-6 were reduced in treated rats. This cytokine has been shown to be increased in different biological fluids in patients with autoimmune disease, particularly RA (Houssiau *et al.*, 1988; Swaak *et al.*, 1988; Hirano *et al.*, 1988), and the level in various inflammatory compartments appears to be a sensitive marker of disease activity.

The protection of bone integrity in the AA rat led us to evaluate SB 203580 in a direct *in vitro* assay of bone resorption, the fetal rat long bone assay. Cytokines such as IL-1 and TNF-α have been shown to stimulate bone resorption *in vitro* and *in vivo* (Gowen and Mundy, 1986; Bertolini *et al.*, 1986; Tashijian *et al.*, 1987; Sabatini *et al.*, 1988), and it was reasonable to expect that a CSAID™ molecule would have a protective effect in this model system. SB 203580 dose-dependently (IC_{50}, 0.6 μM) inhibited PTH-stimulated bone resorption. Although the precise mechanism of action of the compound (and other pyridinyl imidazoles) on bone resorption has not been defined fully, it appears to be related to the compound's cytokine suppressive properties as selective CO, and dual CO/LO inhibitors were inactive in this organ culture system (Votta and Bertolini, 1994).

Another animal model in which TNF-α has been shown to play a predominant role is that of endotoxin-induced shock. We demonstrated previously that SK&F 86002, a dual inhibitor of arachidonic acid metabolism as well as a cytokine inhibitor, could reduce serum TNF-α levels and prolong survival in mouse shock models (Badger *et al.*, 1988). In addition, we were able to demonstrate that antibodies to mouse TNF-α could protect mice against endotoxin-induced shock in mice that were sensitized with *Proprionibacterium acnes* (Badger *et al.*, 1989). SB 203580, a more selective cytokine inhibitor with reduced inhibitory activity on LO and CO, reduced serum TNF-α in LPS/D-gal-sensitized mice and improved their survival at high doses.

It is clear that SB 203580 is a potent inhibitor of IL-1 and TNF-α *in vitro* and that it is pharmacologically active in a number of animal models *in vivo*. The question of whether such a potent cytokine inhibitor would be immunosuppressive as well as having anti-inflammatory activity has been addressed by examining its activity *in vivo* in mice immunized with OVA. Apart from partial inhibition of specific antibody levels against OVA, there was no suppression of OVA-specific T-cell proliferation, an allogeneic response or of mitogen (Con A)-induced proliferative responses. These results and those reported previously with the dual inhibitor of arachidonic acid metabolism, SK&F 86002 (Lee *et al.*, 1993; Reddy *et al.*, 1994), show clearly that these compounds do not have overt immunosuppressive activity.

Mechanistically, it is not clear at the present time to what extent the beneficial effects of SB 203580 are due to suppression of TNF-α production or suppression of cytokine signaling. Given that SB 203580 has been shown, at least *in vitro*, to be effective in inhibiting cytokine signaling leading to either cytokine production or other downstream effects, it is safe to assume that *in vivo*, the compound may induce its antiarthritic activity *via* both the inhibition of cytokine production and action. As an *in vitro* example, SB 203580 has been shown to block IL-6 production in L929 cells stimulated with TNF-α (Beyaert *et al.*, 1996).

The pharmacological profile that we have described here for SB 203580, a potent CSBP/p38 kinase inhibitor, would appear to be one that would be desirable for an antiarthritic therapeutic agent. Despite numerous attempts over the years to design drugs with therapeutic potential for RA, there is still a real need for more effective, less toxic treatments to control the progression of this disease. Most therapies, although supplying symptomatic relief, do not alter the progression of bone and cartilage destruction in the affected joints. In recent years, it has become clear that a multitude of cytokines contribute to the overall inflammatory and bone destructive sequelae that occur in RA, and that targeting one or more of these cytokines could modulate the disease (Arend and Dayer, 1955; Elliott and Maini, 1995). TNF-α has emerged as a cytokine of pivotal importance in the disease process and inhibition of the production and/or effects of this cytokine is a rational therapeutic strategy (Feldmann *et al.*, 1994; Brennan *et al.*, 1995). Indeed, ongoing studies are demonstrating the efficacy of treatment of RA with monoclonal antibodies to TNF-α in RA patients (Elliott *et al.*, 1993; Maini *et al.*, 1995). A small molecular weight orally active cytokine inhibitor with the pharmacological profile described in this manuscript could well provide significant beneficial effects in this disease.

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Send reprint requests to: Dr. Alison M. Badger, Associate Director, Department of Cellular Biochemistry, SmithKline Beecham Pharmaceuticals, 709 Swedeland Rd., P.O. Box 1539. King of Prussia, PA 19406-0939.